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# Cellular and Hormonal Factors Influencing Monocyte Differentiation to Osteoclastic Bone-Resorbing Cells\*

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## ABSTRACT

Osteoclasts are multinucleated cells which form by fusion of circulating mononuclear hemopoietic precursors. The nature of these precursor cells and the roles bone stromal cells and hormonal factors play in their differentiation to osteoclasts are unknown. We cocultured adherent murine blood monocytes (nonspecific esterase and F4/80 positive; tartrate-resistant acid phosphatase negative) with osteoblastic and fibroblastic stromal cell lines in the presence of  $2 \times 10^{-8}$  M 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>]. Tartrate-resistant acid phosphatase and calcitonin (CT) receptor-positive osteoclastic cells, which formed numerous resorption pits *in vitro*, were noted after only 4 days in coculture with UMR106 osteoblast-like cells. Resorption was seen

in cocultures to which as few as 100 peripheral blood mononuclear cells had been added. 1,25-(OH)<sub>2</sub>D<sub>3</sub> and contact with live bone stromal cells were absolute requirements for monocyte differentiation into bone-resorbing cells. Both salmon CT (5 IU/ml) and prostaglandin E<sub>2</sub> ( $10^{-6}$  M) significantly inhibited bone resorption.

Thus, a significant proportion of the peripheral blood mononuclear cells in the monocyte fraction are capable of differentiating into cells showing the cytochemical and functional characteristics of osteoclasts. The presence of specific hormonal [1,25-(OH)<sub>2</sub>D<sub>3</sub>] and bone stromal cell elements is necessary for this process to occur; the resultant resorption can be modulated by known inhibitors of bone resorption, CT and prostaglandin E<sub>2</sub>. (Endocrinology 134: 2416-2423, 1994)

THE OSTEOCLAST (OC) is a highly specialized multinucleated bone-resorbing cell formed by fusion of circulating mononuclear cells of hemopoietic origin (for reviews, see Refs. 1 and 2). OCs form part of the mononuclear phagocyte system (MPS) and share many morphological, cytochemical, and functional characteristics with tissue macrophages and macrophage polykaryons. Although both OCs and macrophages are derived from the pluripotential hemopoietic stem cell, the point at which macrophage and OC differentiation pathways diverge is not certain. It has been proposed that OCs have a cell lineage distinct from that of monocytes and macrophages (3). Conversely, there is much evidence to show that OCs and macrophages share a common myeloid progenitor (4, 5) and that more committed cells of the monocyte-macrophage lineage also give rise to OCs (6-9). Tissue macrophages and macrophage polykaryons are derived from blood monocytes (10), but whether a significant proportion of this same population of circulating cells can also become committed to OC differentiation, possibly on encountering some stimulus found only at the bone surface, is not known.

Multinucleated cells showing phenotypic and functional features of OCs have been generated from bone marrow hemopoietic cells in long term cultures (11). These cell populations contain abundant primitive hemopoietic precursor cells and stromal cell elements. Until recently, it was considered that mature MPS cells from extraskeletal tissues could

not differentiate into OCs (12, 13). However, it has now been shown that monocytes and macrophages (9, 14, 15) can be stimulated to differentiate *in vitro* into OC-like cells that resorb cortical bone. For this to occur, mononuclear phagocytes need to be cocultured with particular bone-derived stromal cell lines in the presence of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>]. These findings suggest that monocytes and macrophages may differentiate into OCs on encountering particular stromal cell-derived stimuli at a site on the surface of bone. 1,25-(OH)<sub>2</sub>D<sub>3</sub> and macrophage colony-stimulating factor have been shown to be important factors in OC differentiation (16-18), but the role other systemic and local hormonal factors play is unclear.

In this study we have further characterized the process of differentiation of peripheral blood mononuclear cells (PBMCs) of the monocyte fraction to osteoclastic bone-resorbing cells *in vitro*. In particular, we have sought to determine some details of the mechanism by which stromal cells promote monocyte/macrophage differentiation to bone-resorbing cells and to estimate the proportion of PBMCs capable of differentiating into osteoclastic cells. In addition, we have investigated whether such differentiation can be influenced by known inhibitors of osteoclastic bone resorption, calcitonin (CT) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

## Materials and Methods

### Media and sera

Incubations were performed in  $\alpha$ -Minimum Essential Medium ( $\alpha$ MEM; Gibco, Paisley, Scotland) supplemented with glutamine (2 mM), benzyl penicillin (100 IU/ml), streptomycin (100 mg/ml  $\alpha$ MEM), and 10% fetal calf serum (FCS; batch F91120, TechGen, London, United Kingdom).

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Kingdom;  $\alpha$ MEM-FCS).  $\alpha$ MEM alone or Hanks' Balanced Salt Solution (HBSS; Flow, Irvine, United Kingdom) was used for cell isolation.

#### Stromal cell lines

Cloned hormone-responsive UMR106 rat osteoblast-like cells and ROS 17/2.8, a rat osteosarcoma-derived cell line, were obtained from Prof. T. J. Martin (Melbourne, Australia); ST2, a preadipocytic marrow stromal cell line, was obtained from the Riken cell bank (Tsukuba, Japan); L929, a fibroblast-like cell line, was obtained from the Sir William Dunn School of Pathology (Oxford, United Kingdom).

#### Hormones

1,25-(OH)<sub>2</sub>D<sub>3</sub>, a gift from Roche (Welwyn Garden City, United Kingdom), was dissolved in absolute alcohol and stored at -20°C. Salmon CT, a gift from Armour Pharmaceuticals (Eastbourne, United Kingdom), was dissolved in 0.05% sodium chloride-0.2% sodium acetate with 0.1% BSA and stored in 25-IU aliquots at -70°C. PGE<sub>2</sub> (Sigma, Poole, United Kingdom) was dissolved in absolute alcohol and stored in aliquots of  $5 \times 10^{-3}$  M at -20°C.

#### Preparation of murine PBMC

After heart puncture of MF1 mice, blood was collected and diluted 1:4 in HBSS; this was layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ), then centrifuged (693  $\times$  g), washed, and resuspended in HBSS. The number of cells in the resulting suspension of PBMC was counted in a hemocytometer. Monocytes were purified from this cell suspension by adhesion to substrate (cortical bone slices or glass coverslips) and characterized as described below.

#### Preparation of stromal cell-PBMC cocultures on human cortical bone slices and coverslips

Human cortical bone slices (4  $\times$  3 mm), prepared as previously described (19), were placed in 6-mm wells. Stromal cells (10<sup>4</sup>; UMR106, L929, ROS 17/2.8, or ST2) were added to each well and cultured on bone slices for 24 h in  $\alpha$ MEM-FCS. The PBMC suspension was then settled (10<sup>5</sup> cells/well) on the bone slices for 60 min. The bone slices were removed from the 6-mm wells, washed vigorously in  $\alpha$ MEM to remove nonadherent cells, and placed in 16-mm wells containing 1 ml  $\alpha$ MEM-FCS with  $2 \times 10^{-8}$  M 1,25-(OH)<sub>2</sub>D<sub>3</sub>; the latter was replenished every third day of culture. After incubation, the bone slices were removed from culture and processed for scanning electron microscopy (SEM), as described below.

As negative controls, PBMCs (10<sup>5</sup> and 10<sup>6</sup> cells/well) were also added to bone slices and coverslips that had not been previously seeded with stromal cells. Medium and 1,25-(OH)<sub>2</sub>D<sub>3</sub> were again replenished every 3 days. As a further negative control, stromal cells alone were similarly incubated for 14 days on bone slices, then processed for SEM, as described below.

For each experiment, monocytes were also prepared on 6-mm glass coverslips; these were either seeded 24 h earlier with stromal cells or unseeded. These coverslip cultures were maintained under the same conditions as those for bone slices.

#### Adherence to substrate of PBMCs differentiating to bone-resorbing cells

To further characterize bone resorption by adherent PBMCs that were present in the above cocultures, bone slices were seeded with PBMCs (10<sup>5</sup> cells/well) in the absence of stromal cells. PBMCs were settled for 60 min, vigorously washed in  $\alpha$ MEM-FCS, and placed in 16-mm wells containing  $\alpha$ MEM-FCS with added 1,25-(OH)<sub>2</sub>D<sub>3</sub> and incubated for up to 3 days alone on bone slices. Half of the bone slices were then removed and placed in 6-mm wells containing 100  $\mu$ l  $\alpha$ MEM-FCS. Four  $\times$  10<sup>4</sup> UMR106 cells were added to each of the 6-mm wells containing these bone slices. After 120 min, bone slices were removed and placed in 16-mm wells containing  $\alpha$ MEM-FCS with added 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Bone slices were then cultured for 7 and 14 days, as described above.

#### Estimation of the proportion of PBMCs differentiating into bone-resorbing cells

A suspension of PBMCs (10<sup>6</sup> cells/ml), prepared in  $\alpha$ MEM-FCS, was sequentially diluted in  $\alpha$ MEM-FCS to produce suspensions of 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, and 10<sup>2</sup> cells/ml. One hundred microliters of each suspension were added to 10 wells; 6 wells contained bone slices that had been previously seeded with UMR106 cells, and 4 wells contained glass coverslips that had not been seeded with stromal cells. In this way, cocultures on bone slices or glass coverslips were prepared containing 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, or 10<sup>1</sup> PBMCs/well. Each suspension was settled on the bone slices or coverslips for 60 min and then washed in  $\alpha$ MEM to remove nonadherent cells. The coverslips were fixed in 10% formalin and stained with toluidine blue. The number of mononuclear cells on each coverslip was either counted directly (10<sup>3</sup>, 10<sup>2</sup>, and 10<sup>1</sup> cells/well) or calculated using an eyepiece graticule, and the number of cells in a minimum of 20 random high power fields was counted (10<sup>4</sup>, 10<sup>5</sup> cells/well). The cocultures on bone slices were incubated for 14 days in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, as described above. Cells were then removed from the bone slices by trypsin and NH<sub>4</sub>OH and prepared for examination by SEM, as described below. The bone slices were scored as to whether they showed evidence of resorption, i.e. contained at least 1 unequivocal resorption pit. This experiment was repeated 4 times.

#### Time course of bone resorption in adherent PBMC-stromal cell coculture

PBMCs (10<sup>5</sup>) were added to 10<sup>4</sup> UMR106 cells that were settled 24 h earlier on bone slices. After washing, these bone slices were placed in 16-mm wells containing  $\alpha$ MEM-FCS with added  $2 \times 10^{-8}$  M 1,25-(OH)<sub>2</sub>D<sub>3</sub> and cultured for 2, 3, 4, 5, 6, 7, and 8 days before being fixed and prepared for examination by SEM (six bone slices per time point of incubation; five experiments).

#### Characterization of stromal cell influence in adherent PBMC-stromal cell cocultures

To determine whether UMR106 stromal cells release a soluble factor that stimulates differentiation to bone-resorbing cells, 10<sup>5</sup> PBMCs were settled on bone slices either devoid of stromal cells or covered with L929 (10<sup>4</sup> cells/well) that had been seeded on the bone slices 24 h previously. After washing, the bone slices were placed into fresh wells containing  $\alpha$ MEM-FCS and 1,25-(OH)<sub>2</sub>D<sub>3</sub>. A Falcon cyclopore insert (Becton Dickinson, Oxford, United Kingdom) was added which contained a 9-mm diameter porous membrane (pore size, 0.45  $\mu$ m<sup>2</sup>) on which had been seeded UMR106 cells (4  $\times$  10<sup>4</sup> cells/well). The chamber membrane pores were large enough to allow transfer of soluble factors but no physical contact between the cells. These bone slices were cultured for 14 and 21 days before fixation. Positive controls consisted of cocultures of UMR106 cells with PBMCs isolated from the same animal as that described above. In addition, bone slices containing adherent PBMCs alone or adherent PBMC/L929 cell cocultures were cultured in UMR106-conditioned medium containing 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

To test whether, as has been reported (20), contact with devitalized fixed stromal cells could initiate OC differentiation, bone slices were seeded with 4  $\times$  10<sup>4</sup> UMR106 cells in 6-mm wells and cultured overnight, as described above. These bone slices containing UMR106 cells were then removed, rinsed thoroughly in HBSS, and placed in sterile PBS containing 0.25% glutaraldehyde; they were then removed from the glutaraldehyde, washed in HBSS, then rinsed in PBS containing 0.1% BSA. The bone slices thus prepared were again soaked in HBSS before being returned to fresh 6-mm wells. PBMCs (10<sup>5</sup> or 10<sup>6</sup>/well) were settled onto each bone slice. Nonadherent cells were removed by washing, and the bone slices were placed in 1 ml  $\alpha$ MEM-FCS with  $2 \times 10^{-8}$  M 1,25-(OH)<sub>2</sub>D<sub>3</sub> and incubated for 21 days. The bone slices were then processed for examination by SEM, as described below.

#### Effects of CT and PGE<sub>2</sub> on bone resorption by adherent PBMC-stromal cell cocultures

PBMCs settled onto bone slices seeded 24 h previously with UMR106 stromal cell were placed after washing in 16-mm wells containing

$\alpha$ MEM-FCS with  $2 \times 10^{-8}$  M 1,25-(OH)<sub>2</sub>D<sub>3</sub> and either 10<sup>-6</sup> M PGE<sub>2</sub> (replenished every 3 days) or 5 IU salmon CT (replenished daily). Cultures were incubated for 7, 11, and 14 days before fixation and examination by SEM. For each incubation period, 12 bone slices were examined in each of 3 experiments.

To determine whether, as has been reported (20), PGs could be substituted for 1,25-(OH)<sub>2</sub>D<sub>3</sub> in OC progenitor-stromal cell coculture systems, monocyte-stromal cell cocultures prepared as described above (10<sup>5</sup> PBMCs with 10<sup>4</sup> UMR106 cells) were transferred to 16-mm wells containing either 10<sup>-6</sup> M PGE<sub>2</sub> or  $2 \times 10^{-8}$  M 1,25-(OH)D<sub>3</sub> and incubated for 14 days before fixation and examination by SEM.

#### Histochemical characterization of isolated and cultured cells

For histochemistry, cell preparations were fixed in 2.5% paraformaldehyde in PBS and stained for acid phosphatase using naphthol AS-B1 phosphate (Sigma) as a substrate in the presence or absence of 50 mM tartrate; the reaction product was stained with hexazotized pararosaniline (Sigma). For nonspecific esterase (NSE) staining, the cells cultured on coverslips were fixed in paraformaldehyde vapor and incubated in naphthylacetate (Sigma), which was used as a substrate; the reaction product was colored with fast-blue salt (Sigma). The presence of F4/80 antigen on adherent PBMCs was determined using an indirect immunoperoxidase technique, as previously described (21, 22).

#### Demonstration of CT receptors

The presence of CT receptors was assessed by autoradiography using [<sup>125</sup>I]salmon CT, as previously described (23). PBMCs and stromal cells (either UMR106 or ST2 cells) were cocultured on 13-mm coverslips for 1 h at 22°C in  $\alpha$ MEM-FCS containing 0.15% BSA with 0.2 nM [<sup>125</sup>I]CT (Amersham International, Aylesbury, United Kingdom). After thus allowing the labeled CT to bind, cells were washed with cold  $\alpha$ MEM, fixed for 10 min in 2% glutaraldehyde-10% formalin solution, and dried. Nonspecific binding was assessed in the presence of an excess amount of unlabeled CT (300 nM). The coverslips were then dipped in K-5 photographic emulsion and processed for autoradiography. Negative controls consisted of stromal cells alone; positive controls consisted of adherent cultured rabbit OCs.

#### Preparation of bone slices for examination by SEM and estimation of bone resorption

Cells were removed from the bone slices by immersion in 0.25 M ammonium hydroxide overnight. The bone slices were passed through graded alcohols to absolute alcohol, then allowed to air dry before being sputter-coated with gold and examined in a Philips SEM505 scanning electron microscope.

In experiments in which cells were cultured for 7 days or less, resorption pits were often relatively few in number and small in area; therefore, in this case only the number of pits on each bone slice was counted. For comparison, the number of pits was also counted on bone slices on which cells had been cocultured for 14 days. There was considerably more resorption in both 11- and 14-day cultures, and this greater resorption was better assessed by measuring the surface area of the bone resorbed. This was performed by point counting using reflected light microscopy; the proportion of area resorbed was examined using a 100  $\times$  100 eyepiece graticule, as previously described (18).

#### Statistical analysis

Each series of experiments was repeated at least five times. Results are expressed as the mean  $\pm$  SEM, and significance was determined using parametric (Student's *t* test) and nonparametric (Mann-Whitney *U* test) statistical tests. Results analyzed by both methods showed the same levels of significance (indicated in Fig. 6).

#### Results

##### Characteristics of isolated PBMCs and bone resorption in coculture

Adherent mononuclear leukocytes isolated from peripheral blood were characterized as monocytes on the basis that they were (>90%) NSE and F4/80 positive, but entirely negative for tartrate resistant acid phosphatase (TRAP) and CT receptors. After the monocytes were cocultured with UMR106 or ST2 cells in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 7 days, not only were large numbers of NSE- and F4/80-positive cells present, but scattered CT receptor- and TRAP-positive cells as well as TRAP-positive cell clusters were also noted (Figs. 1 and 2). The UMR106 cells also weakly expressed CT receptors, as has previously been described (24), but these were not seen in ST2-monocyte cocultures. In 14-day cocultures, larger clusters of TRAP- and CT receptor-positive cells were noted. In contrast, F4/80-positive cells were randomly scattered throughout the 14-day cocultures, with no F4/80-positive cell clusters evident.

Resorption was only noted on bone slices on which monocytes had been cocultured with UMR106 or ST2 stromal cells in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Fig. 3). No resorption occurred when 1,25-(OH)<sub>2</sub>D<sub>3</sub> was omitted from these cocultures. Culture of either monocytes or stromal cells alone on cortical bone slices [in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>] also did not result in bone resorption. Bone resorption was not seen in cocultures of monocytes and L929 fibroblast-like cells, cocultures of monocytes and ROS 17/2.8 cells, or monocytes alone in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

Time-course experiments showed that bone resorption first occurred after monocytes had been in coculture with UMR106 stromal cells for 4 days, when pits (mean, 4.46  $\pm$  1.39) were seen on bone slices. Over an 8-day period, a rise in the number of resorption pits was noted; a large increase occurred at 6 days of incubation (Fig. 4).

Bone resorption was seen when monocytes were isolated and cultured alone on bone slices for between 1-3 days before the addition of UMR106 cells. This indicates that some of those cells in the monocyte fraction that differentiate to form bone-resorbing cells are strongly adherent to the bone substrate and can survive *in vitro* for up to 3 days before the addition of stromal cells that stimulate them to undergo differentiation.

The proportion of monocytes capable of differentiating in coculture to bone-resorbing cells was determined by sequential dilution of the PBMC suspension added to the bone slices. The results of five experiments are summarized in Fig. 5. This shows that bone resorption was observed when only 10<sup>2</sup> PBMCs were present in the suspension added to bone slices; more than half of the bone slices cultured with 10<sup>3</sup> PBMCs showed evidence of bone resorption. However, as not all cells present in such a suspension remain adherent to substrate after washing, an estimate of the number of adherent monocytes was gained by settling an equal volume of identically diluted PBMC suspensions onto 6-mm coverslips. After washing, cultures were fixed and stained with toluidine blue, and the number of adherent cells on each coverslip

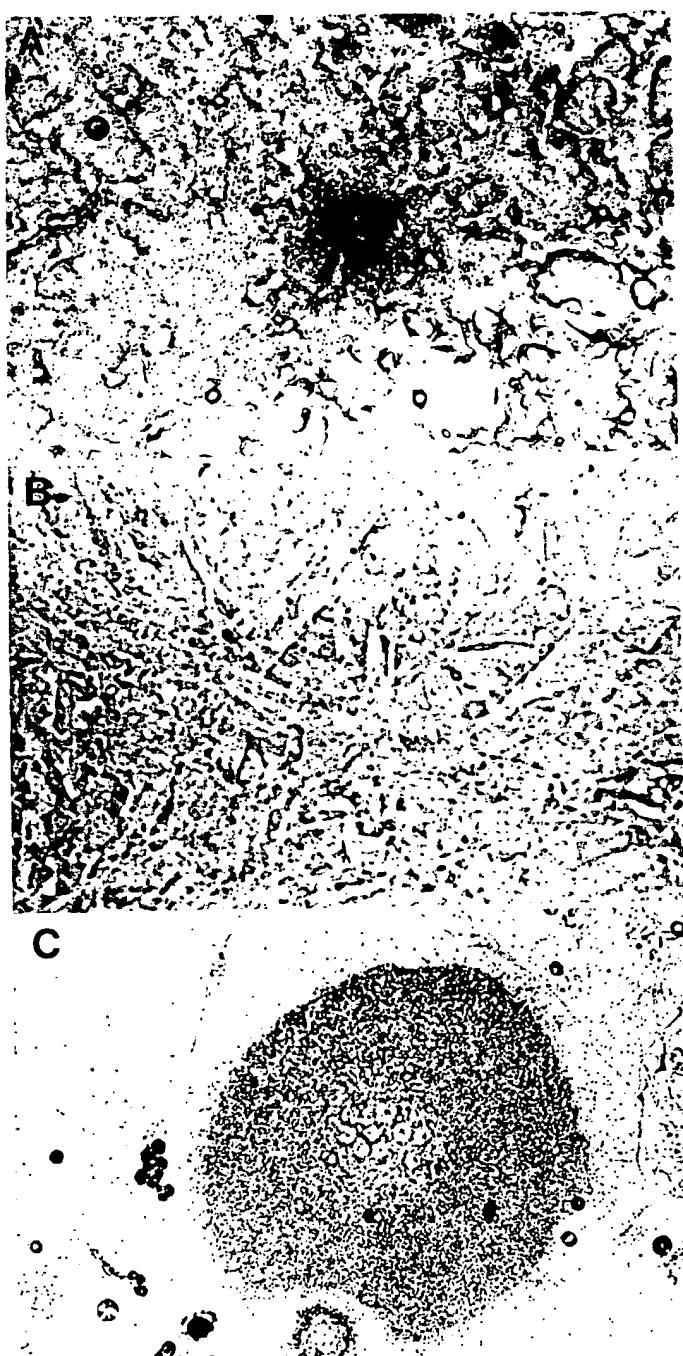


FIG. 1. Expression of CT receptors demonstrated by autoradiographic detection of bound [ $^{125}\text{I}$ ]salmon CT. A, CT receptor-positive cells in cocultures of monocytes with ST2 cells after 7 days of incubation in the presence of  $1,25\text{-(OH)}_2\text{D}_3$  (magnification,  $\times 200$ ). B, For comparison, coculture of monocytes with ST2 cells [7-day incubation in the presence of  $1,25\text{-(OH)}_2\text{D}_3$ ] in which cells were incubated with [ $^{125}\text{I}$ ]salmon CT in the presence of excess (5 IU/ml) unlabeled salmon calcitonin is shown (magnification,  $\times 200$ ). C, Positive control; cultured OCs derived from the femur of a young rabbit (magnification,  $\times 400$ ).

was counted. This showed that when  $10^5$  PBMCs were added to the coverslips,  $8.7 \pm 1.3 \times 10^3$  adherent cells/well remained after washing;  $10^4$  PBMCs gave  $6.8 \pm 0.56 \times 10^2$  cells/well,  $10^3$  PBMCs gave  $104 \pm 17.4$  cells/well, and  $10^2$

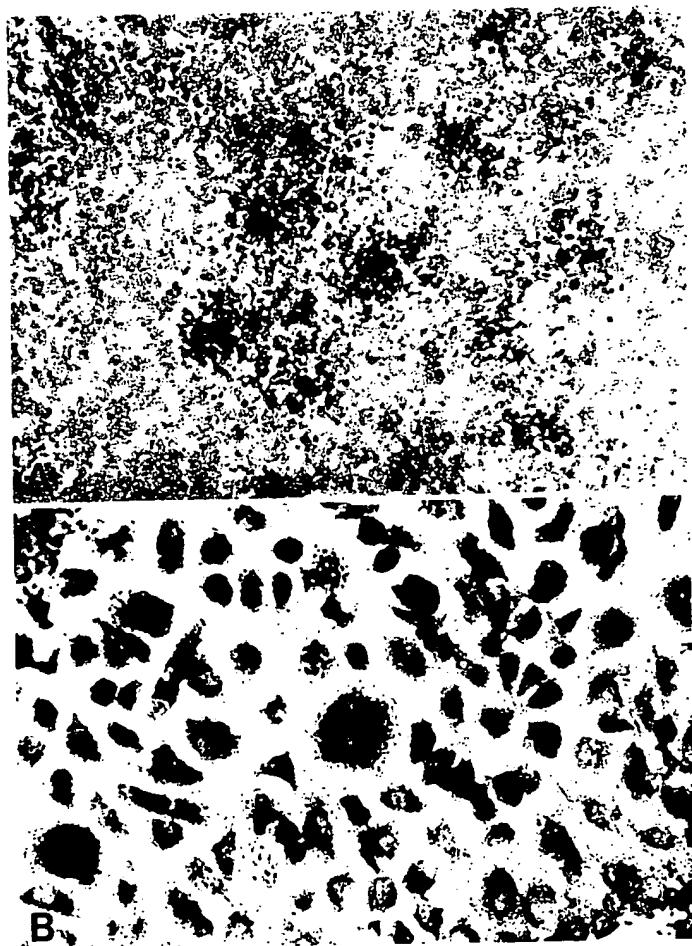


FIG. 2. Cytochemical staining for TRAP in cocultures of murine monocytes and UMR106 cells incubated for 7 days in the presence of  $1,25\text{-(OH)}_2\text{D}_3$ , showing several discrete clusters of TRAP-positive cells (A; magnification,  $\times 100$ ) and a high power view of a cluster of TRAP-positive cells, including a large multinucleated cell (B, center; magnification,  $\times 400$ ).

PBMCs gave  $5.3 \pm 0.86$  cells/well. The average surface area of the bone slices was approximately  $12 \text{ mm}^2$ , so assuming a uniform distribution of PBMCs throughout the well, approximately 3000 monocytes remained on each bone slice seeded with  $10^5$  PBMCs; for  $10^4$  PBMCs, approximately 230 monocytes remained; for  $10^3$  PBMCs, approximately 35 monocytes remained; and for  $10^2$  PBMCs, approximately 2 monocytes remained.

#### *Stromal cell influence on bone resorption*

When conditioned medium from UMR106 or ST2 stromal cells was added to monocytes cultured alone on bone slices [with  $1,25\text{-(OH)}_2\text{D}_3$ ], no resorption resulted. Similarly, conditioned medium added to monocytes cocultured on bone slices with L929 cells [with  $1,25\text{-(OH)}_2\text{D}_3$ ] also failed to show evidence of bone resorption. Diffusion chamber experiments in which UMR106 cells were cultured on a membrane filter separated from monocytes that were incubated alone on a bone slice [in the presence of  $1,25\text{-(OH)}_2\text{D}_3$ ] resulted in no resorption. In further experiments, UMR106 cells covering

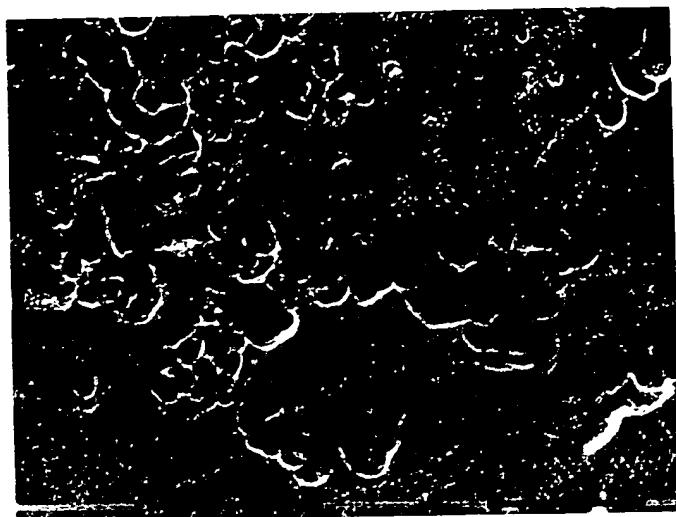


FIG. 3. Extensive lacunar resorption seen on bone slices after 14-day coculture of murine monocyte and UMR106 cells in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (■ = 100  $\mu$ m).

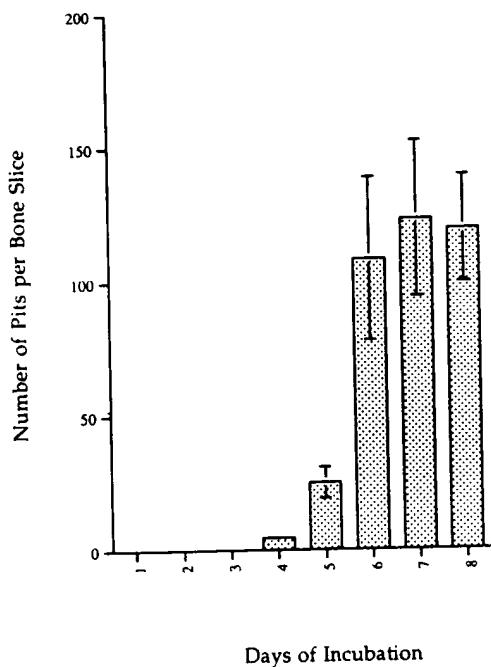


FIG. 4. Number of resorption pits (per bone slice) in murine monocyte-UMR106 stromal cell cocultures incubated in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for between 1-8 days.

the bone slices were fixed in glutaraldehyde before the addition of the monocytes; when these cocultures were incubated in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for up to 2 weeks, no resorption resulted.

#### Influence of CT and PGE<sub>2</sub> on bone resorption

When monocyte-UMR106 cocultures were incubated in the presence of salmon CT, there was significantly less bone resorption after 7 days in coculture. Inhibition of resorption was also noted in 11- and 14-day cultures, although the

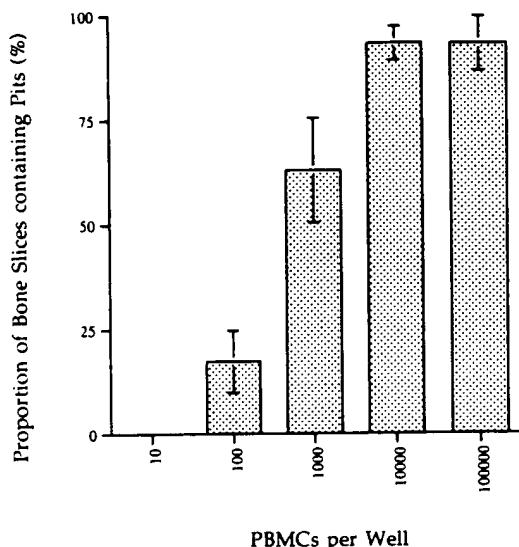


FIG. 5. Proportion of bone slices showing evidence of bone resorption when 10<sup>6</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, or 10<sup>1</sup> PBMCs were added to wells containing bone slices preseeded with UMR106 cells and incubated for 14 days.

effect here was less marked. There was also a striking decrease in resorption when PGE<sub>2</sub> was added to monocyte/UMR106 cocultures; this effect persisted throughout the 14-day period (Fig. 6).

It was also noted that when PGE<sub>2</sub> was substituted for 1,25-(OH)<sub>2</sub>D<sub>3</sub>, during the incubation of the monocyte-UMR106 cocultures on bone slices, no resorption resulted.

#### Discussion

OC precursors are known to be derived from the pluripotential hemopoietic stem cell and to be present in the circulation. However, neither their precise identity nor the hormonal and cellular influences that control their differentiation into mature OCs are known. In this study we have shown that a significant proportion of PBMCs characterized phenotypically as monocytes can differentiate *in vitro* to become osteoclastic cells capable of extensive lacunar bone resorption. This differentiation took place only under specific conditions of hormonal and stromal cell stimulation. Known inhibitors of osteoclastic activity, *viz.* CT and PGE<sub>2</sub>, resulted in a significant decrease in the bone resorption resulting from monocyte-stromal cell cocultures.

Mononuclear cells isolated from peripheral blood were characterized as monocytes on the basis that they were NSE and F4/80 positive and entirely negative for TRAP and CT receptors. As previous studies have shown, monocytes by themselves did not form resorption pits on bone slices (12). However, after being cultured with UMR106 or ST2 stromal cells [in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>], some of these cells showed features characteristic of OCs, including TRAP and CT receptor positivity; most strikingly, they also developed the functional ability to produce numerous resorption pits on bone slices after only 4 days in culture. Monocytes settled on bone slices for 3 days before the addition of the UMR106 cells showed a similar capacity to resorb bone. Thus, like

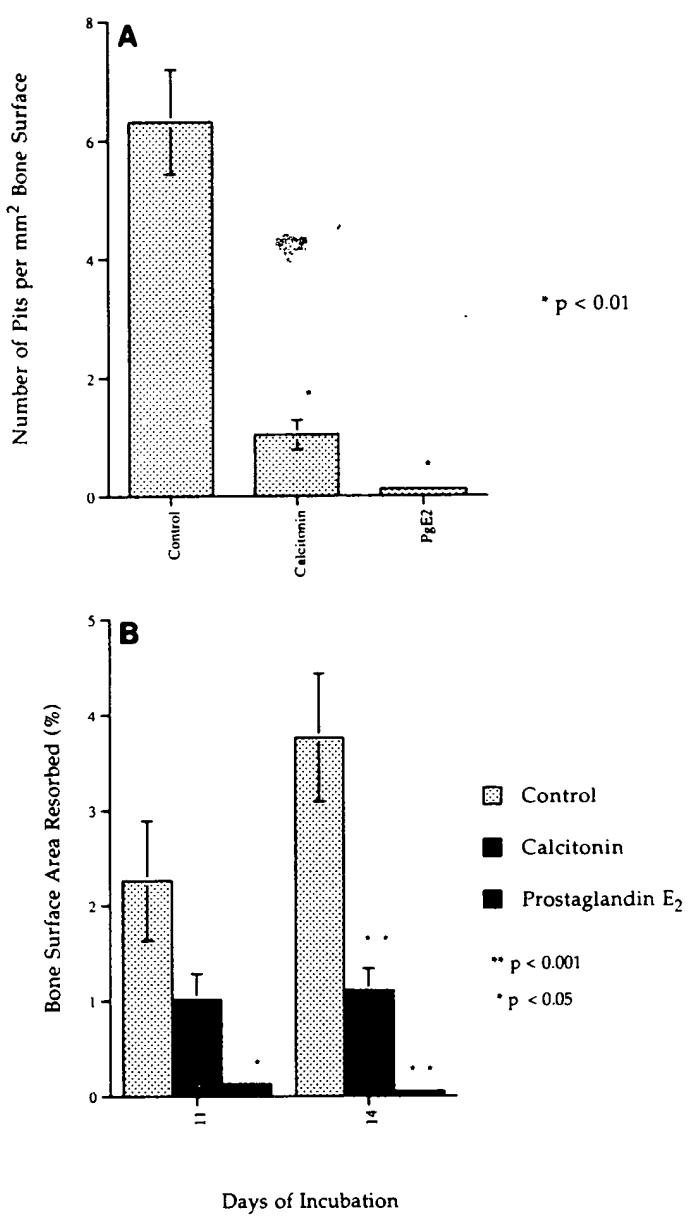


FIG. 6. Bone resorption in murine monocyte-UMR106 stromal cell cocultures incubated in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> alone (control), 1,25-(OH)<sub>2</sub>D<sub>3</sub> plus 5 IU/ml CT, or 1,25-(OH)<sub>2</sub>D<sub>3</sub> plus 10<sup>-6</sup> M PGE<sub>2</sub> showing the amount of resorption after 7 days of incubation, measured as number of pits per mm<sup>2</sup> bone slice area (A), and the amount of resorption after 11 and 14 days of incubation, measured as the percentage of the bone slice area resorbed (B).

OCs (and other MPS cells), they adhere strongly to bone and are relatively long-lived in culture on the bone surface. Bone resorption frequently resulted when cell suspensions containing as few as 1000 PBMCs (of which ~35 remained adherent to the bone slice) were cocultured with UMR106 cells and 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and was also noted when as few as 100 PBMCs were present in the cell suspension added to the bone slices. This indicates that although not all cells in the monocyte fraction are capable of differentiating into osteoclastic bone-resorbing cells, the proportion capable of doing so is surprisingly high. That such a relatively large subpop-

ulation of PBMCs can differentiate into osteoclastic cells *in vitro* would argue against such cells being determined precursors of the OC alone and is consistent with the notion that OCs and macrophages share a common monocyte precursor.

Although it was originally postulated that monocytes as a whole cell population form part of the OC lineage and are the direct precursors of OCs, a major objection to this has been the inability of monocytes to produce resorption pits when cultured on cortical bone slices (12). Our results and the work of Udagawa *et al.* (9) would indicate that the crucial element lacking in these experiments was the presence of specific hormonal and stromal cell elements required to support osteoclastic differentiation. There is much evidence to favor OC origin from cells of the monocyte/macrophage lineage. OC-like cells have been formed *in vitro* from immature cells of this lineage, including granulocyte-macrophage colony-forming cells (4-6). Avian peripheral blood monocytes have also been observed to fuse with OCs *in vitro* (8) and have been shown to differentiate into OCs in long term culture (25). In addition, not only blood monocytes, but also macrophages derived from extraskeletal tissues, have been shown to be capable of differentiation to OC-like cells in coculture with bone stromal cells (9, 14, 15).

The concept that cells of the monocyte fraction are capable of directly differentiating into OCs is supported by the work of Baron *et al.* (26), who showed *in vivo* what appears to occur *in vitro* in our experiments, namely that macrophage-like mononuclear cells that contain NSE (but not TRAP) appear at resorbing surfaces before mononuclear cells that contain either TRAP or both TRAP and NSE. These observations would suggest that mononuclear OC precursor cells undergo several steps in differentiation to become functional OCs. Monocytes are known to be chemotactically attracted to certain constituents of the bone matrix (27). 1,25-(OH)<sub>2</sub>D<sub>3</sub> is known to induce human monocyte to macrophage maturation *in vitro* (28) and to promote the expression of several factors that are important in both macrophage and OC differentiation (29-31). These include fusion of monocytes and macrophages, substrate adhesion, phagocytic activity, the ability to degrade devitalized bone particles, and expression of lineage-restricted macrophage antigens.

Hemopoietic spleen and marrow cell cultures, in which osteoclastic cells develop *in vitro*, require long term incubation before resorption pits are formed (11, 30). Using cultures of mouse spleen cells with osteoblastic cells, Tanaka *et al.* (16) showed that OC progenitors undergo proliferation in the first 4 days of culture and differentiate thereafter into TRAP and CT receptor-positive OC-like cells. However, they did not correlate the onset of these OC markers with the onset of bone resorption. Our results suggest that formation of functional osteoclastic cells from cells of the monocyte fraction similarly involves two distinct phases. We found that an identical minimum period of 4-day coculture of PBMCs with UMR106 cells was required before resorption pits were first formed. TRAP-positive cells were also seen at this time. Numerous discrete clusters of TRAP- and CT receptor-positive cells, which may have represented small colonies of

proliferating OC precursors, were also noted in longer term monocyte-UMR106 cocultures.

Whether an accessory stromal cell element is necessary for the generation of OCs from OC precursors is controversial (2). Our results are in agreement with those of previous studies which have shown that in cocultures of spleen-derived hemopoietic cells with stromal cell lines, OC differentiation is supported only by stromal cell lines showing phenotypic characteristics of marrow stromal cells or osteoblast-like cells (30). Some of these stromal cell lines, such as ST2, are relatively unstable in their ability to promote OC differentiation (data not shown). The UMR106 rat osteosarcoma cell line (which has not previously been demonstrated to support OC generation) has some advantage in this regard in that it appears to be relatively stable, supporting OC differentiation over many passages. In addition, UMR106 osteoblast-like cells show phenotypic characteristics of the cell type lining the bone surface where OCs form and are required to function (32). Although Collins and Chambers (20) have shown that some glutaraldehyde-fixed stromal cell lines can promote OC formation from spleen-derived hemopoietic cells, we have shown that contact with live UMR106 cells is necessary for differentiation to bone-resorbing cells. Our investigations thus far have also not provided any evidence that these osteoblast-like cells release a soluble factor that promotes OC differentiation *in vitro*.

The effects of PGs on the skeleton are complex, with both stimulation and inhibition of bone resorption reported (1). Collins and Chambers (20) have shown that PGE<sub>2</sub> can be substituted for 1,25-(OH)<sub>2</sub>D<sub>3</sub> and that it can promote OC formation in murine hemopoietic cell cultures; their observations were based on experiments with a coculture system that employed spleen hemopoietic cells and marrow stromal cells. In the monocyte-stromal cell coculture system that we have employed, the addition of identical concentrations of PGE<sub>2</sub> resulted in strong inhibition of bone resorption after 7, 11, and 14 days of incubation. The different stromal and hemopoietic cell types employed in their studies could account for the paradoxical effects of PGE<sub>2</sub>. A similar, but less striking, inhibitory effect on bone resorption was seen with CT, which, like PGE<sub>2</sub>, is known to directly inhibit OC activity (3).

CT is known to induce a decrease in OC numbers both *in vitro* and *in vivo* and to decrease OC activity and resorption pit formation (33-35). PGE<sub>2</sub> is known to inhibit the formation of OC-like cells in long term cultures of human marrow cells stimulated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> (36). PGs of the E series have also been shown to inhibit the proliferation of mononuclear phagocytes, including those of macrophage colony-stimulating factor-dependent cell lines (37, 38). In the bone resorption assay that we have employed it is not possible to determine precisely whether CT and PGE<sub>2</sub> act to inhibit the generation of osteoclastic cells from mononuclear precursors or whether their main effect is to inhibit the formation of bone resorption pits by the osteoclastic cells formed in coculture. Escape of mature OCs from the inhibitory effect of these hormones is a well recognized phenomenon (33, 39); indeed, in the case of PGE<sub>2</sub>, this inhibition is typically followed by (osteoblast-

mediated) stimulation of bone resorption. In contrast, our results show that these hormones have a persistent inhibitory effect on bone resorption when monocytes are cocultured with the osteoblast-like cells for both short (7-day) and long (11- and 14-day) term incubation periods. The timing, degree, and persistence of this inhibition of bone resorption in our cocultures would argue against this effect being achieved solely by a reduction in resorption pit formation by osteoclastic cells. The dramatic inhibition of bone resorption observed after PGE<sub>2</sub> administration could also have been mediated through an effect on stromal cells. PGE<sub>2</sub> is known to increase cAMP production in UMR106 and other stromal cells, which supports differentiation to osteoclastic cells in similar coculture systems (2, 40); on the other hand, ROS 17/2.8 cells do not show a similar response to PGs of the E series (41). Moreover, PGE<sub>2</sub> is known to influence collagenase release and proliferation of UMR106 cells (42, 43), both of which may influence the differentiation or function of bone-resorbing cells.

In conclusion, we have shown that cells showing the cytochemical and functional characteristics of OCs can be produced from the monocyte fraction of PBMC. 1,25-(OH)<sub>2</sub>D<sub>3</sub> and specific stromal cells are necessary for this differentiation to occur, and known inhibitors of bone resorption, namely CT and PGE<sub>2</sub>, strongly inhibit this process. This coculture model is likely to be rewarding in studying the roles of other hormonal mediators, pharmacological agents, and cellular and tissue factors in osteoclastic bone resorption.

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